Pergamon

Insect Biochemistry and Molecular Biology 30 (2000) 729–737

Insect Biochemistry and Molecular Biology

www.elsevier.com/locate/ibmb

Terminal steps in JH biosynthesis in the honey bee (*Apis mellifera* L.): developmental changes in sensitivity to JH precursor and allatotropin

Anna Rachinsky [a,*], Stephen S. Tobe [b], Mark F. Feldlaufer [a]

[a] *Bee Research Laboratory, USDA-ARS, Bldg. 476, BARC-East, Beltsville, MD 20705, USA*
[b] *Department of Zoology, University of Toronto, 25 Harbord St, Toronto, Ontario, Canada M5S 3G5*

Received 31 October 1999; received in revised form 31 December 1999; accepted 25 January 2000

Abstract

Juvenile hormone (JH) is considered the prime endogenous signal for the induction of queen development in honey bees (*Apis mellifera* L.). At the beginning of the last (5th) larval stadium, worker corpora allata synthesize less JH than queen corpora allata as a consequence of a limited production of JH precursors and a caste- and stage-specific block of the terminal step in JH biosynthesis. As previously shown, the *Manduca sexta* allatotropin stimulates JH biosynthesis in honey bee corpora allata in a dose-dependent and reversible manner, but can not overcome the stage-specific block in the terminal step of JH biosynthesis that is typical for worker early 5th instars. In experiments with *M. sexta* allatotropin and with the JH precursor farnesoic acid, we found characteristic stage-specific differences in their effects on JH biosynthesis. From the end of the spinning stage on, corpora allata could be stimulated by farnesoic acid to a much higher extent than in earlier developmental stages, suggesting a sudden increase in epoxidase activity. *Manduca sexta* allatotropin, however, stimulated corpora allata activity until the end of the spinning stage, at which time the corpora allata become suddenly insensitive. These data suggest that in worker larvae, important changes in the regulation of the terminal enzymatic steps in JH biosynthesis occur at the transition from the spinning stage to the prepupal stage. However, the analysis of in vitro activities of the involved enzymes, *O*-methyltransferase and methyl farnesoate epoxidase, remained inconclusive.

Keywords: JH biosynthesis; Allatotropin; Farnesoic acid; *O*-methyltransferase; Methyl farnesoate epoxidase; Caste development; *Apis mellifera*

1. Introduction

Induction of queen development in the honey bee, *Apis mellifera* L., is principally governed by juvenile hormone (JH) (Nijhout and Wheeler, 1982; Hartfelder and Engels, 1998). At the beginning of the last (5th) larval stadium, i.e. during the feeding stage, worker corpora allata (CA) synthesize considerably less JH than queen CA, mainly as a consequence of a limited production of the JH precursor methyl farnesoate (Rachinsky and Hartfelder, 1990). This period of differential CA activity corresponds to caste-specific differences in JH titer and JH whole body content (Rachinsky et al., 1990; Rembold, 1987). In addition to a limited production of JH precursor, caste- and stage-specific differences in the regulation of the terminal steps in JH biosynthesis contribute to the observed differences in JH production. JH III was found to be the only JH homologue present in all developmental stages of the honey bee (Trautmann et al., 1974; Hagenguth and Rembold, 1978). The terminal two steps in JH III biosynthesis generally are: (1) the methylation of farnesoic acid (FA) to methyl farnesoate (MF) catalyzed by an *O*-methyl transferase; and (2) an epoxidation of MF to JH III by the enzyme methyl farnesoate epoxidase (Tobe and Stay, 1985). In many insect species, exogenous farnesoic acid can be utilized by corpora allata in vitro to enhance rates of JH III production (Tobe and Stay, 1985). In honey bee larvae, there are caste-, stage- and sex-specific differences in the utilization of exogenous farnesoic acid. By experimentally increasing the concentration of FA,

* Corresponding author. Present address: Department of Entomology, Kansas State University, 123 West Waters Hall, Manhattan, KS 66506, USA. Tel.: +1-785-532-4723; fax: +1-785-532-6232.
E-mail address: arachins@oz.oznet.ksu.edu (A. Rachinsky).

0965-1748/00/$ - see front matter
PII: S0965-1748(00)00044-8

JH release rates could be stimulated several-fold in CA from queen feeding stage 5th instars, in CA from male feeding stage 5th instars, and in CA from queen and worker prepupae (Rachinsky and Hartfelder, 1991; Hartfelder et al., 1993). In CA from worker feeding stage 5th instars, however, an increase in FA concentration resulted in an enormous accumulation of the immediate JH precursor methyl farnesoate in the glands without causing a significant increase in JH release rates (Rachinsky and Hartfelder, 1991), suggesting a block in the terminal step of JH biosynthesis. Under unstimulated conditions, there is no such accumulation of methyl farnesoate in feeding stage worker CA (Rachinsky and Hartfelder, 1990). Thus, two different mechanisms seem to be responsible for the low rates of JH production by worker CA during this developmental stage: (1) a limitation in the production of JH precursor; and (2) an additional mechanism that causes a rate-limitation in the terminal step of JH biosynthesis. In the search for allatotropic factors consideration should be given to the possibility that in feeding stage worker glands, JH production may be limited at different levels, each of which may require a separate mechanism for its stimulation.

Only one stimulatory CA regulator has been purified and characterized to date from heads of pharate adult *Manduca sexta* (Kataoka et al., 1989). *Manduca sexta* allatotropin (Mas-AT) only stimulated adult CA in vitro, but did not affect the activity of CA from larval or pupal stages. In another lepidopteran species, *Heliothis virescens*, Mas-AT also stimulated JH biosynthesis in adult CA, but it had no effect on CA activity in adults of several other insect species (Kataoka et al., 1989). Several other putative peptides with allatotropic properties have been partially characterized (Granger et al., 1984; Gadot and Applebaum, 1985; Lehmberg et al., 1992; Lorenz and Hoffmann, 1995; Bogus and Scheller, 1996). In honey bee larvae, there is evidence for the presence of allatotropic material in the brain (Rachinsky, 1996) but to date, complete characterization has not been accomplished. Recently we showed that *M. sexta* allatotropin stimulates JH biosynthesis in honey bee larvae (Rachinsky and Feldlaufer, 2000). The allatotropin stimulated larval honey bee CA to produce significantly more of the JH precursor methyl farnesoate, an effect that could be reversed by removing the stimulatory peptide from the incubation medium. Our results indicated that there may be structurally related peptides in honey bee larvae, regulating caste- and stage-specific production of JH.

In this study, we investigated the stage-specific effects of Mas-AT and farnesoic acid on honey bee corpora allata during the developmental period in which caste development occurs. We further describe the results of analysis of CA homogenates from different developmental stages for the activities of *O*-methyltransferase and methyl farnesoate epoxidase.

2. Materials and methods

2.1. Chemicals

[^{3}H]S-adenosyl-L-methionine (SAM) (458.8 GBq/mmol) and L-[^{14}C-methyl]-methionine (2.14 GBq/mmol) were purchased from New England Nuclear (Boston, MA). [12-^{3}H] Methyl farnesoate (244.2 GBq/mmol) was a gift from Dr G.D. Prestwich. The farnesoic acid used for experiments was either a gift from Dr F.C. Baker, or synthesized from *trans*,*trans*-farnesol (Aldrich, Milwaukee, WI) using a protocol described by Caliezi and Schinz (1949). Part of the newly synthesized FA was converted into its methyl ester, methyl farnesoate, by reaction with diazomethane; identity and purity of the product were confirmed by GC-MS. *Manduca sexta* allatotropin was purchased from Sigma (St Louis. MO). All chemicals used for the preparation of the incubation medium for corpora allata and for the enzyme assays were obtained from Sigma (St Louis, MO) or Aldrich (Milwaukee, WI). All glassware used for enzyme assays was treated with polyethyleneglycol (MW 20,000) to prevent adsorption of the substrates to the reaction vials (Feyereisen et al., 1981).

2.2. Honey bee larvae

Worker larvae were collected from queenright *A. mellifera* colonies maintained at apiaries in Beltsville, MD, and in Toronto, Canada. Worker larvae of the 4th (L4) and the 5th (L5) stadia were distinguished by the differences in maximum width of their head capsules which measured 0.999±0.003 mm (mean±SEM; n=100) for L4 and 1.634±0.004 mm (mean±SEM; n=272) for L5, respectively. As described previously (Rachinsky et al., 1990), the 5th larval stadium was divided into nine substages of which five were chosen for experiments. Those were: (i) small and (ii) medium-sized feeding stage larvae that were characterized by body weight (L5F1: 30–60 mg; L5F2: 61–110 mg), (iii) early and (iv) late spinning stage larvae that could be easily distinguished by checking the contents of their gut under a dissecting microscope (L5S1: entire gut filled with yellowish pollen mass; L5S3: gut completely voided), and (v) early prepupae (L5PP1) which could be classified by measuring the tibio-tarsal length of the developing hind legs (1.40–1.99 mm).

For both methyltransferase and epoxidase assays, worker larvae of the substages L5F2 and L5PP1 were selected. These developmental stages differ markedly with regard to CA activity (Rachinsky and Hartfelder, 1990), JH titer (Rachinsky et al., 1990) and utilization of exogenous farnesoic acid by the CA (Rachinsky and Hartfelder, 1991). For assays on epoxidase activity, male honey bee larvae (drones) of the developmental stage

L5F2 (characterized by body weight according to Hartfelder et al., 1993) were included in the study.

2.3. Radiochemical in vitro assay

Dissection of corpora allata and incubation were carried out in a saline and an incubation medium, respectively, specifically designed for larval honey bees (Rachinsky and Hartfelder, 1998). Following the dissection, each pair of CA was immediately transferred to 50 μl unlabeled, methionine-free incubation medium and preincubated at room temperature for 10–20 min prior to the radiochemical in vitro assay (Pratt and Tobe, 1974; Tobe and Pratt, 1974). Each pair of glands was then transferred into 25 μl radiolabeled control medium (50 μM L-[^{14}C-methyl-] methionine) or into 25 μl radiolabeled medium that contained farnesoic acid (40 μM), or *M. sexta* allatotropin (100 μM), or both FA and Mas-AT. Glands were incubated for 2 h at 34.0°C with gentle shaking. Both preincubation and incubation were carried out in a humid chamber, in U-shaped acrylic plastic wells (LINBRO® 96-well tissue culture plates; 0.2 ml well capacity; ICN Biomedicals, Aurora, OH).

At the end of the incubation period, CA and incubation medium were separated. As a measure of JH biosynthesis, the rates of JH released into the medium were determined by using a rapid partition radiochemical assay (Feyereisen and Tobe, 1981). The medium was extracted with 300 μl isooctane in 6×50 mm borosilicate tubes. An aliquot taken from the isooctane hyperphase was assayed by liquid scintillation spectrometry to determine JH release. Measurements were corrected by subtracting the radioactivity found in the isooctane phase from blank incubations (radiolabeled medium without CA).

The CA were transferred into glass culture tubes (12×75 mm) and extracted separately. Extraction, separation and quantification of in vitro labeled JH and MF was carried out according to Feyereisen (1985); thin-layer chromatography (TLC) was carried out using a hexane/ethyl acetate (3:1) solvent system. The separate analysis of CA and incubation medium yields information on juvenile hormone release rates (expressed as pmol JH/pair CA×h^{-1}), as well as on the amounts of juvenile hormone and methyl farnesoate present in the CA (expressed as pmol JH or MF/pair CA).

2.4. Enzyme preparation

Corpora allata were dissected from feeding stage worker larvae (L5F2), from worker prepupae (L5PP1), or from male feeding stage 5th instars (male L5F2). CA were homogenized on ice with a glass–glass homogenizer (Kontes, Vineland, NJ) in 100 mM sodium phosphate buffer, pH 7.2, supplemented with 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM 2-mercaptoethanol and 1% bovine serum albumine (BSA) (slightly modified from Feyereisen et al., 1981). After centrifugation at 10,000***g*** for 15 min at 4°C, the supernatant was used as the *O*-methyltransferase preparation. For the epoxidase preparation, the supernatant derived from a centrifugation at 5000***g*** for 30 min, was used.

2.5. Methyltransferase assay

The assay for *O*-methyltransferase activity followed largely the procedures of Feyereisen et al. (1981) and Wang et al. (1994). Each two CA equivalents of the enzyme preparation (equivalent to one pair of CA) were incubated for 2 h in a water bath at 34°C, in a final volume of 100 μl 100 mM sodium phosphate buffer (pH 7.2; with 1 mM EDTA; 1 mM 2-mercaptoethanol and 1% BSA) supplemented with 0.45 μM [^{3}H]-SAM, 9.55 μM unlabeled SAM (10 μM total SAM concentration) and 40 μM farnesoic acid. To establish optimum assay conditions, FA concentrations from 10 to 160 μM, and incubation time from 30 min to 4 h were tested.

The reaction was stopped by the addition of 200 μl methanol. After the addition of 20 μg each of unlabeled carriers of JH III and methyl farnesoate, the mixture was extracted with chloroform (2×1 ml). The combined chloroform phases were passed through anhydrous Na_2SO_4, then dried under N_2, redissolved in diethyl ether and applied to TLC plates (Merck silica gel 60 F_{254}, plastic sheets). After focussing twice with methanol, the samples were separated in a solvent system of toluene:ethyl acetate:acetic acid (85:15:0.5 v/v). The bands corresponding to JH III and methyl farnesoate were cut out and assayed for radioactivity by liquid scintillation spectrometry. Enzyme activity was expressed as radioactivity (dpm in the respective band) per CA equivalent.

2.6. Epoxidase assay

An appropriate amount of enzyme preparation (six CA equivalents per incubation) was incubated in a final volume of 100 μl with 0.5 mM β-nicotinamide adenine dinucleotide phosphate (β-NADPH), 5 mM glucose-6-phosphate, 0.25 units glucose-6-phosphate dehydrogenase, and [12-^{3}H]methyl farnesoate (Feyereisen et al., 1981, as modified by Wang et al., 1994). The concentration of labeled methyl farnesoate was 0.15 μM. The mixture was incubated for 45 min at 30°C in a water bath. The reaction was stopped by addition of 100 μl 1% Na-EDTA and 200 μl methanol. Unlabeled carriers (each 20 μg JH III and methyl farnesoate) were added to the reaction mixture prior to extraction with chloroform (2×750 μl). All other steps were as described for the methyltransferase assay.

2.7. Statistics

Mean rates (±SEM) of JH release were calculated by measuring 4–25 individual pairs of corpora allata per group, or by determining JH and MF contents in 3–11 individual extractions. For enzyme assays, 3–9 individual measurements were made. Statistical analyses included analysis of variance (ANOVA) followed by Tukey–Kramer Multiple Comparisons Test. Differences were considered significant when the P value was <0.05.

3. Results

3.1. Stage-specific differences in farnesoic acid utilization

Because there are developmental changes in JH titer and JH biosynthesis by corpora allata of late larval instar worker honey bees (Rachinsky et al., 1990; Rachinsky and Hartfelder, 1990) that may, in part, be the result of stage-specific differences in the regulation of the terminal steps in JH biosynthesis (Rachinsky and Hartfelder, 1991), we analyzed the in vitro utilization of farnesoic acid by CA at the end of the 4th and throughout the 5th stadium. In addition, in the same developmental stages, the effect of *M. sexta* allatotropin on the terminal steps in JH biosynthesis was studied. The results are shown in Figs. 1 and 2. Experimentally elevated FA concentrations (Fig. 1) caused a highly significant increase in methyl farnesoate production in corpora allata from 4th instars, feeding stage 5th instars (L5F1, L5F2) and early spinning stage larvae (L5S1). This led to a large accumulation of MF in glands from these stages. In the late spinning stage (L5S3) and in the prepupal stage (L5PP1) no significant increase in methyl farnesoate content of the CA occurred. Rather, in these last mentioned developmental stages, juvenile hormone production was stimulated by exogenous FA to a much higher extent than in the earlier developmental phases. In L5S3 and L5PP1, JH release reached peak values of 3.6 pmol/pair CA×h^{-1}, whereas, until the early spinning stage, FA-stimulated JH release did not exceed 1.7 pmol/pair CA×h^{-1}. In all developmental stages in this study (except for L5S1), FA treatment significantly increased the very low JH content of the corpora allata (<0.5 pmol/pair CA in control incubations). From the data on intraglandular MF content and on JH release, it appears that the CA acquire the ability to produce and release significantly higher amounts of JH from exogenous farnesoic acid only in spinning stage 5th instars.

3.2. Stage-specific differences in the effects of M. sexta allatotropin on JH biosynthesis

Similar developmental changes as observed in the above-described experiments with farnesoic acid

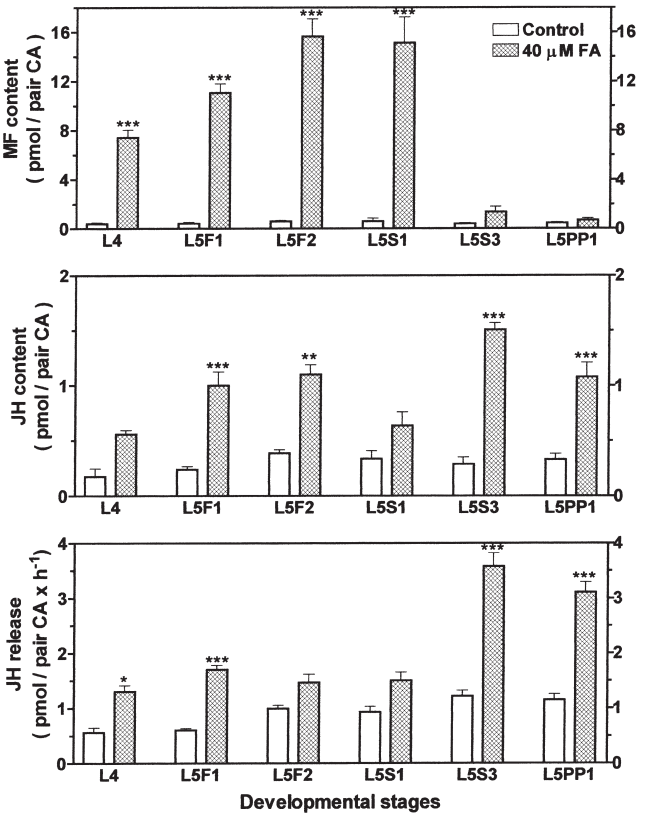

Fig. 1. Developmental changes of farnesoic acid (FA) effects on intraglandular contents of methyl farnesoate (MF) and JH, and on JH release from worker honey bee corpora allata. Corpora allata were incubated in vitro with 40 μM of the JH precursor farnesoic acid. Bars represent the means±SEM of 3–11 replicates for MF and JH content, and of 6–25 replicates for JH release rates. Asterisks denote significant differences from respective control values (ANOVA; * $P\leq0.05$; ** $P\leq0.01$; *** $P\leq0.001$). L4=worker 4th instars; L5F1, L5F2=worker feeding stage 5th instars; L5S1, L5S3=worker spinning stage 5th instars; L5PP1=worker early prepupae.

occurred in honey bee corpora allata stimulated with *M. sexta* allatotropin (Fig. 2). From the 4th stadium until the beginning of the spinning stage of the 5th stadium, Mas-AT treatment enhanced methyl farnesoate production, which resulted in a highly significant accumulation of MF in the corpora allata. However, no MF accumulation occurred in late spinning stage larvae and in prepupae. Juvenile hormone content of the CA was elevated under Mas-AT-stimulated conditions from the 4th stadium until the end of the spinning stage of the 5th stadium, but not in prepupae. The same was true for JH release rates: they were unaffected by Mas-AT in prepupae, but significantly stimulated from the 4th stadium until the end of the spinning stage of the 5th stadium. These results suggest a change in sensitivity to Mas-AT at the end of the spinning stage.

Application of both Mas-AT and FA to corpora allata from feeding stage 5th instars resulted in the same effects as FA alone (Fig. 3). In both cases, the most prominent effect was a large increase in intraglandular

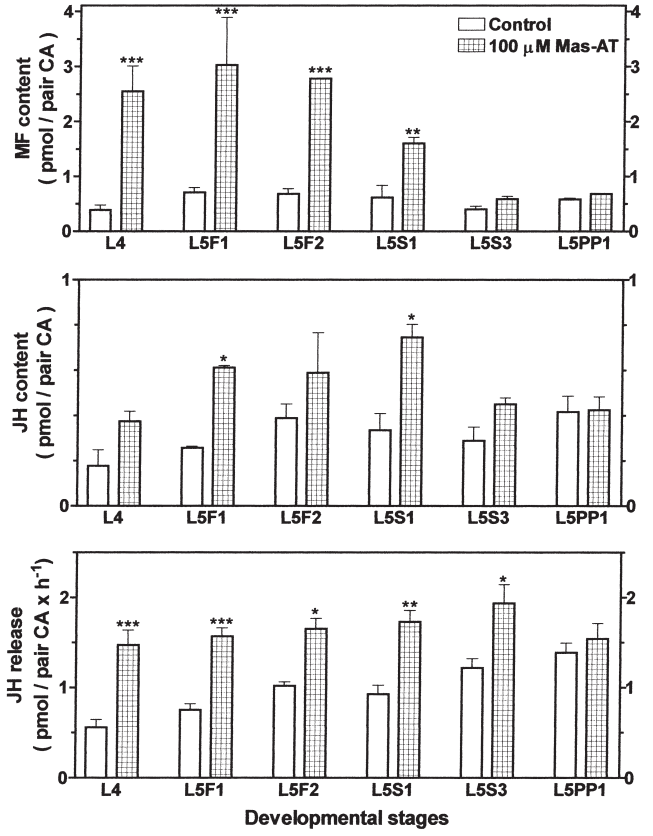

Fig. 2. Stage-specific effects of *M. sexta* allatotropin (Mas-AT) on intraglandular contents of methyl farnesoate (MF) and JH, and on JH release from worker honey bee corpora allata. Corpora allata were incubated in vitro with 100 μM Mas-AT. Bars represent the means±SEM of three replicates for MF and JH content, and of 4–18 replicates for JH release rates. Asterisks denote significant differences from respective control values (ANOVA; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). L4=worker 4th instars; L5F1, L5F2=worker feeding stage 5th instars; L5S1, L5S3=worker spinning stage 5th instars; L5PP1=worker early prepupae.

MF. In both cases, JH release rates were elevated as compared to untreated control incubations. However, JH release from corpora allata treated with Mas-AT and FA was not higher than from CA that had been treated with either FA or Mas-AT alone.

3.3. Activity of O-methyltransferase and methyl farnesoate epoxidase

Our experiments with FA and Mas-AT suggest stage-specific differences in sensitivity of honey bee corpora allata to allatotropic factors, as well as developmental changes in the utilization of JH precursors. This may involve developmental changes in the activity of the enzymes that catalyze the two terminal steps in JH biosynthesis. For this reason, we studied the activity of the *O*-methyltransferase and of the methyl farnesoate epoxidase in CA homogenates of feeding stage 5th instars and prepupae.

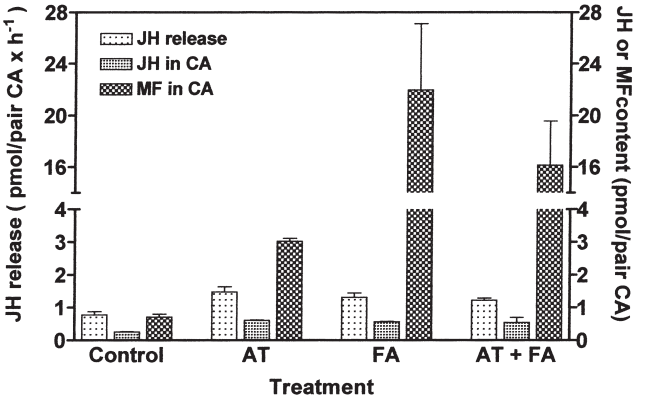

Fig. 3. Effects of *M. sexta* allatotropin (Mas-AT) and farnesoic acid (FA) on corpora allata activity in worker feeding stage 5th instars (L5F2). Corpora allata were incubated in vitro with 100 μM Mas-AT or 40 μM FA or a combination of both. Medium and CA were extracted separately to determine JH release rates, as well as intraglandular contents of methyl farnesoate (MF) and JH. Bars represent the means±SEM of three replicates for MF and JH content, and of 4–5 replicates for JH release rates.

Under our assay conditions and in both developmental stages used for this study, *O*-methyltransferase activity was linear during an incubation period of 120 min (Fig. 4). Thus, for all further investigations an incubation time of 2 h was chosen. As a consequence of the rather crude extraction method for the enzyme, JH III was also produced but in much lower quantities. This suggests a slight contamination of the cytosolic fraction with microsomes carrying epoxidase activity. *O*-methyltransferase preparations were used within 24 h of their preparation, because longer storage at 4°C caused a dramatic loss in enzyme activity (Table 1). As shown for CA homogenates from worker feeding stage 5th instars, *O*-methyltransferase activity increased in a dose-dependent manner with increasing FA concentrations (10–160 μM FA;

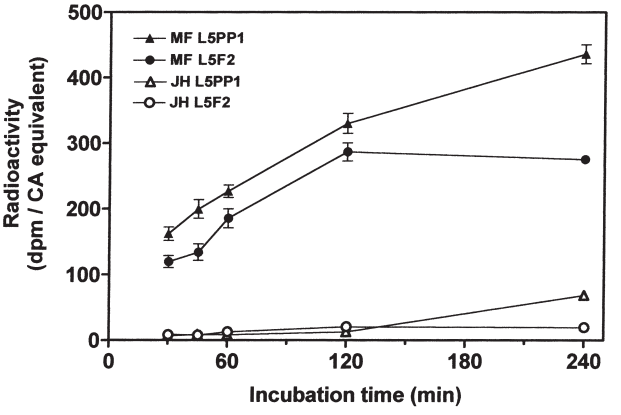

Fig. 4. Time course of *O*-methyltransferase activity in homogenates from worker feeding stage 5th instars (L5F2) and prepupae (L5PP1). Each two CA equivalents of the enzyme preparation were used per assay. The filled symbols stand for methyl farnesoate (MF), the open symbols for JH produced by the respective CA homogenates. Each data point represents the mean±SEM of 3–6 determinations.

Table 1
Loss of methyl transferase (MTF) activity in CA homogenates of feeding stage worker larvae (L5F2) during storage at 4°C

Storage time (d)	MTF activity (dpm/CA equivalent) mean±SEM	*n*	Loss of MTF activity (%)
1	185.58±14.30	3	
3	59.02±3.86	5	68.2
5	26.19±3.23	9	85.9
8	10.53±0.90	5	94.3

Fig. 5). But even at high substrate concentrations (40–160 μM FA), no differences between *O*-methyltransferase activity in feeding stage 5th instars and in prepupae could be detected (Fig. 5).

The activity of the methyl farnesoate epoxidase was determined in three developmental stages that differed in their ability to utilize FA for JH production in vitro (Rachinsky and Hartfelder, 1991; Hartfelder et al., 1993) and, thus, were expected to show differences in epoxidase activity. The enzyme activities measured in these stages differed from our expectations: epoxidase activity was higher in feeding stage 5th instars than in prepupae or in male feeding stage 5th instars (Fig. 6).

4. Discussion

4.1. Stage-specific changes in responsiveness of honey bee corpora allata to farnesoic acid

It has been previously reported that the utilization of exogenous farnesoic acid by corpora allata from worker feeding stage larvae differs from that by corpora allata

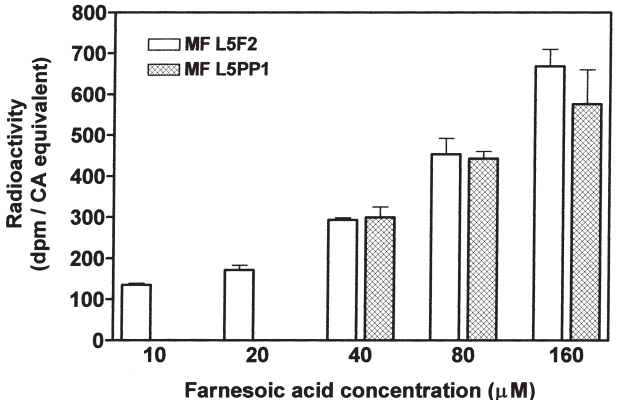

Fig. 5. Total *O*-methyltransferase activity in CA homogenates from worker feeding stage 5th instars (L5F2) and prepupae (L5PP1) at different concentrations of farnesoic acid (FA). For L5F2, FA concentrations from 10–160 μM were used, for L5PP1 only the higher FA concentrations (40–160 μM) were used. Each two CA equivalents of the enzyme preparation were incubated for 2 h at 34°C. Bars represent the means±SEM of 4–5 replicates.

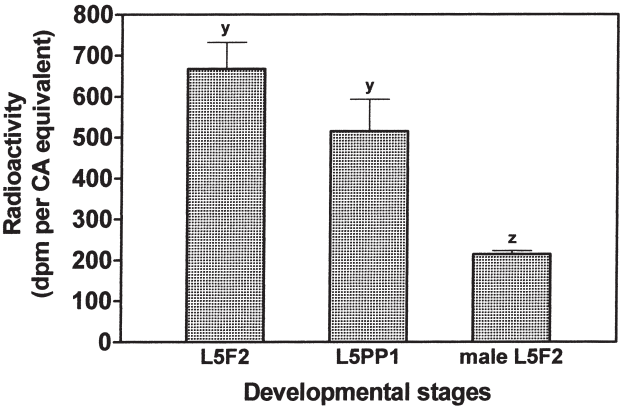

Fig. 6. Methyl farnesoate epoxidase activity in CA homogenates from worker feeding stage 5th instars (L5F2) and prepupae (L5PP1), and from male feeding stage 5th instars (male L5F2). Each six CA equivalents of the enzyme preparation were incubated for 45 min at 30°C. Bars represent the means±SEM of 4–5 replicates. Letters (*y*,*z*) indicate significant difference in epoxidase activity (ANOVA; $P \leq 0.05$).

of worker prepupae (Rachinsky and Hartfelder, 1991). In this study we were able to pinpoint the middle of the spinning stage as the developmental phase in which the switch from high methyl farnesoate accumulation/low increase in JH release to no MF accumulation/significant increase in JH release occurs. This result may be explained by stage-specific changes in enzyme activity. The activity of the methyl farnesoate epoxidase appears to be low until the beginning of the spinning stage of the 5th stadium. It then increases at the end of the spinning stage, leading to an elevated conversion of methyl farnesoate to JH and, therefore, to less intraglandular accumulation of the precursor and a greater stimulation of JH release rate (Fig. 1). This period during which methyl farnesoate epoxidase activity is low in worker larvae corresponds precisely with a postulated JH-sensitive period for queen induction in honey bees (Nijhout and Wheeler, 1982; Nijhout, 1994). As previously pointed out by Rachinsky and Hartfelder (1991), the low methyl farnesoate epoxidase activity that occurs in worker early 5th instars in concert with low JH precursor production may be regarded as a safeguard mechanism that ensures the generation of caste-specific differences in JH titer during a critical stage of honey bee caste development. This may be a mechanism to prevent the development of queen/worker intermediates, which are not tolerated in a honey bee colony. Such queen/worker intermediates develop under certain experimental conditions, e.g. as a result of topical JH applications to worker 5th instars (Wirtz, 1973; Zdarek and Haragsim, 1974) or following transfer of worker 5th instars into queen cells and exposure to a queen-specific diet after a critical period during which they become committed to worker development (Weiss, 1978; Dedej, 1998).

In only a few other insect species does methyl farne-

soate accumulation in corpora allata occur under certain experimental conditions. Specific inhibition of methyl farnesoate epoxidase activity by 1,5-disubstituted imidazoles resulted in an increase in intraglandular MF levels in *Diploptera punctata* (Unnithan et al., 1995). Elevated concentrations of exogenous FA led to an accumulation of MF in corpora allata from *Periplaneta americana* (Pratt et al., 1975). In *Gryllus bimaculatus*, MF accumulation was observed after treatment of the glands with JH III, with FA, or with a combination of FA and high K^+ concentrations (Wennauer and Hoffmann, 1988; Klein et al., 1993). In most insect species, however, FA stimulation does not result in MF accumulation, suggesting that the epoxidative capacity of the corpora allata is generally greater than their capacity for the esterification.

4.2. Stage-specific changes in responsiveness of honey bee corpora allata to Mas-AT

Larval honey bee CA seem to be competent to respond to stimulatory peptidergic input. This was shown using partially characterized fractions from larval honey bee brain extract (Rachinsky, 1996) and synthetic Mas-AT (Rachinsky and Feldlaufer, 2000). Mas-AT stimulated CA activity in worker feeding stage 5th instars but not in prepupae. Several synthetic allatostatins of a similar size to Mas-AT (8–15 amino acid residues) had no effect on CA activity when tested at concentrations up to 100 μM, confirming that the Mas-AT effect was a specific allatotropic effect rather than a non-specific stimulation by peptides in general (Rachinsky and Feldlaufer, 2000). Although larval honey bee CA responded only to high concentrations of Mas-AT (10 or 100 μM), the stage-specificity and reversibility characteristics of the Mas-AT effect suggest that there are specific receptors present that bind Mas-AT with low affinity (Rachinsky and Feldlaufer, 2000).

In this study, we show that the sensitivity of honey bee CA to Mas-AT is dependent on the developmental stage of the larvae and, thus, presumably on a changing physiological status of larval CA. There may be similar stage-dependent changes in sensitivity of larval CA to honey bee-specific CA regulators. From the 4th stadium until the beginning of the spinning stage of the 5th stadium, Mas-AT significantly increased MF production in CA from honey bee worker feeding stage larvae to up to 3.03 pmol/pair CA; intraglandular JH was slightly increased to values up to 0.61 pmol/pair CA (Fig. 2). The total amount of intraglandular MF and JH in these Mas-AT-stimulated worker glands was, thus, in about the same range as the total amount of MF and JH in corpora allata of natural queen larvae in the 5th instar feeding stage (Rachinsky and Hartfelder, 1991). In queen corpora allata, however, JH is by far the more prominent molecule (3.78 pmol JH/pair CA versus 1.30 pmol MF/pair CA; data from Rachinsky and Hartfelder, 1991). Intraglandular JH content and JH release were stimulated by Mas-AT until the end of the spinning stage (Fig. 2). The developmental period during which larval worker CA are sensitive to stimulation by Mas-AT corresponds exactly with the period during which larval queen CA synthesize JH at higher rates (Rachinsky and Hartfelder, 1990). We assume that in queen larvae, throughout the first half of the 5th stadium, allatotropic factors are produced, resulting in increased rates of JH biosynthesis. In worker early 5th instars, the production and/or release of allatotropins seems inhibited resulting in low CA activity. However, these CA are still competent to respond to stimulatory peptides such as Mas-AT (this study) or honey bee-specific brain factors (Rachinsky, 1996).

The developmental pattern of sensitivity of worker CA to Mas-AT corresponds to developmental changes in enzyme activity. As noted above, we concluded that methyl farnesoate epoxidase activity might be low during the first half of the 5th stadium until the beginning of the spinning stage. Low epoxidase activity may explain the accumulation of MF in Mas-AT-stimulated corpora allata that occurs during the same developmental period as FA-stimulated MF accumulation. From the 4th stadium until the beginning of the spinning stage of the 5th stadium, Mas-AT-stimulated JH release rates (1.47±0.16 to 1.73±0.13 pmol/pair CA×h^{-1}; Fig. 2) were in the same range as FA-stimulated JH release rates (1.30±0.11 to 1.71±0.07 pmol/pair CA×h^{-1}; Fig. 1). We conclude from these results that Mas-AT is effective in stimulating JH precursor production in worker glands during the first half of the 5th stadium, but as a consequence of limited epoxidase activity the actual JH release rates can obviously not exceed values of about 1.7 pmol/pair CA×h^{-1}. Mas-AT thus only seems to stimulate the early steps in JH biosynthesis but does not overcome the block of the terminal step in JH biosynthesis. This was confirmed with experiments in which CA were exposed to both FA and Mas-AT at the same time (Fig. 3).

Treatment of CA from late spinning stage larvae with Mas-AT did not result in an intraglandular accumulation of methyl farnesoate, but JH release was significantly increased (Fig. 2). According to our results with exogenous FA, methyl farnesoate epoxidase activity may suddenly increase in the late spinning stage. Thus, if the corpora allata are still sensitive to Mas-AT stimulation at this developmental stage, all the MF that is produced may be converted into JH and released into the medium. This may explain why we do not see a MF accumulation but elevated JH release rates (Fig. 2). At the beginning of the prepupal stage, the CA appear to be insensitive to Mas-AT; we could not detect any stimulation of JH or JH precursor production.

Changes in sensitivity to the allatotropic stimulus

could occur as a consequence of quantitative changes in the respective receptors, changes in receptor specificity, modulation of intracellular signal transduction, or modifications on the target site which may alter the effect of Mas-AT-induced intracellular signals on a specific enzymatic step in JH biosynthesis. Even in *M. sexta*, Mas-AT and Mas-AT analogues were only effective in stimulating JH biosynthesis in adult corpora allata but not in larval or pupal CA (Kataoka et al., 1989; Unni et al., 1991; Hebda et al., 1994). Stage-specific differences in sensitivity are a common feature in allatostatin action, peptides that inhibit corpora allata activity (Stay and Woodhead, 1993; Stay et al., 1994). In the case of the cockroach *D. punctata*, sensitivity of the corpora allata to allatostatins can be experimentally manipulated suggesting the existence of humoral regulators (Unnithan and Feyereisen, 1995). The demonstration of differential sensitivity of the glands to regulatory peptides suggests that these modulators may be physiological regulators only at certain developmental stages, likely those in which the glands are most sensitive to the peptides (Stay and Woodhead, 1993).

4.3. Enzyme activity at different developmental stages

The results from Figs. 1–3 indicate that in worker 5th instars, the activity of the terminal step in JH biosynthesis, the epoxidation of methyl farnesoate to JH III, is blocked until the beginning of the spinning stage and that this block abruptly dissipates at the end of the spinning stage. The limitation of the methyl farnesoate epoxidase in the first half of the 5th stadium seems to be a worker-specific feature, since in feeding stage queen larvae (Rachinsky and Hartfelder, 1991) or in male feeding stage 5th instars (Hartfelder et al., 1993), JH release can be stimulated to a high degree in the presence of exogenous farnesoic acid. On the other hand, the activity of the *O*-methyltransferase seems to be very high in worker feeding stage larvae and early spinning stage larvae, resulting in a large accumulation of intraglandular MF under FA-stimulated conditions (Fig. 1). According to the data in Fig. 1, the total amount of MF and JH accumulated in the glands and released into the medium over a 2 h period, is 19.7 pmol/pair CA (L5F2) and 18.8 pmol/pair CA (L5S1) in worker early 5th instars, but is only 10.0 pmol/pair CA (L5S3) and 8.0 pmol/pair CA (L5PP1) for worker late 5th instars. Thus, in worker early 5th instars, *O*-methyltransferase activity appears to be considerably higher than in late 5th instars. From these results we conclude that in intact worker glands, *O*-methyltransferase activity is high throughout the first half of the 5th stadium and lower from the end of the spinning stage on. For the methyl farnesoate epoxidase, however, we postulate that its activity is limited until the beginning of the spinning stage and increases appreciably at the end of the spinning stage.

However, this indirect evidence for stage-specific changes in enzyme activity could not be confirmed by measurement of enzyme activity in corpora allata homogenates prepared from the respective developmental stages. Contrary to our expectations, there were no differences between *O*-methyltransferase activity in CA homogenates from worker feeding stage larvae and from worker prepupae (Fig. 5). In terms of the activity of the methyl farnesoate epoxidase, we expected to find low enzyme activity in CA homogenates from worker stage feeding larvae and higher enzyme activity in worker prepupae or in male feeding stage larvae. Our results, however, did not support this hypothesis. In some insect species, such as *D. punctata* (Wang et al., 1994), there is a good correlation between enzyme activities in CA homogenates and data on MF and JH production in intact glands. In honey bees, however, the value of data for methyl farnesoate epoxidase activity obtained from measurements in CA homogenates seems questionable. Homogenization completely disrupts any compartmentalization within the glands, which may be crucial for the regulation of enzyme activity and result in data that do not reflect enzyme activities in intact glands. Thus, the estimates for enzyme activities obtained from our experiments with exogenous FA and Mas-AT may provide a more reliable picture of developmental changes in the regulation of the two terminal enzymatic steps of JH biosynthesis in honey bee larvae.

Acknowledgements

We thank Ken Wilzer for technical assistance and Karl Lauber, Toronto for supplying honey bees. This research was supported by postdoctoral fellowships to AR from the Deutsche Forschungsgemeinschaft (Ra 549/2-1) and from the Max Kade Foundation, New York and by an operating grant from the Natural Sciences and Engineering Research Council of Canada to SST.

References

Bogus, M., Scheller, K., 1996. Allatotropin released by the brain controls larval molting in *Galleria mellonella* by affecting juvenile hormone synthesis. International Journal of Developmental Biology 40, 205–210.

Caliezi, A., Schinz, H., 1949. Zur Kenntnis der Sesquiterpene und Azulene. Die Cyclisation der Farnesylsäure. Helvetica Chimica Acta 32, 2556–2560.

Dedej, S., 1998. Caste determination is a sequential process: effect of larval age at grafting on ovariole number, hind leg size and cephalic volatiles in the honey bee (*Apis mellifera carnica*). Journal of Apicultural Research 37, 183–190.

Feyereisen, R., 1985. Radiochemical assay for juvenile hormone III biosynthesis in vitro. Methods in Enzymology 111, 530–539.

Feyereisen, R., Pratt, G.E., Hamnett, A.F., 1981. Enzymic synthesis of juvenile hormone in locust corpora allata: evidence for a

microsomal cytochrome P-450 linked methyl farnesoate epoxidase. European Journal of Biochemistry 118, 231–238.
Feyereisen, R., Tobe, S.S., 1981. A rapid partition assay for routine analysis of juvenile hormone released by insect corpora allata. Analytical Biochemistry 111, 372–375.
Gadot, M., Applebaum, S.W., 1985. Rapid in vitro-activation of corpora allata by extracted locust brain allatotropic factor. Archives of Insect Biochemistry and Physiology 2, 117–129.
Granger, N.A., Mitchell, L.J., Janzen, W.P., Bollenbacher, W.E., 1984. Activation of *Manduca sexta* corpora allata in vitro by a cerebral neuropeptide. Molecular and Cellular Endocrinology 37, 349–358.
Hagenguth, H., Rembold, H., 1978. Identification of juvenile hormone 3 as the only JH homolog in all developmental stages of the honey bee. Zeitschrift für Naturforschung C33, 847–850.
Hartfelder, K., Engels, W., 1998. Social insect polymorphism: hormonal regulation of plasticity in development and reproduction in the honeybee. Current Topics in Developmental Biology 40, 45–77.
Hartfelder, K., Tozetto, S.O., Rachinsky, A., 1993. Sex-specific developmental profiles of juvenile hormone synthesis in honey bee larvae. Roux's Archives of Developmental Biology 202, 176–180.
Hebda, C., Yu, J.-H., Bhaskaran, G., Dahm, K.H., 1994. Reactivation of corpora allata in pharate adult *Manduca sexta*. Journal of Insect Physiology 40, 849–858.
Kataoka, H., Toschi, A., Li, J.P., Carney, R.L., Schooley, D.A., Kramer, S.J., 1989. Identification of an allatotropin from adult *Manduca sexta*. Science 243, 1481–1483.
Klein, P.M., Lorenz, M.W., Donglin, H., Hoffmann, K.H., 1993. Age dependency and regulatory properties of juvenile hormone III biosynthesis in adult female crickets, *Gryllus bimaculatus*. Journal of Insect Physiology 39, 315–324.
Lehmberg, E., Ferenz, H.J., Applebaum, S.W., 1992. Maturation and responsiveness to extracts of corpora allata from male *Locusta migratoria* containing allatotropic factors. Zeitschrift für Naturforschung C47, 449–452.
Lorenz, M.W., Hoffmann, K.H., 1995. Allatotropic activity in the suboesophageal ganglia of crickets, *Gryllus bimaculatus* and *Achaeta domesticus* (Ensifera: Gryllidae). Journal of Insect Physiology 41, 191–196.
Nijhout, H.F., 1994. Insect Hormones. Princeton University Press, Princeton, NJ.
Nijhout, H.F., Wheeler, D.E., 1982. Juvenile hormone and the physiological basis of insect polymorphism. Quarterly Review of Biology 57, 109–133.
Pratt, G.E., Tobe, S.S., 1974. Juvenile hormone radiobiosynthesised by corpora allata of adult female locusts in vitro. Life Sciences 14, 575–586.
Pratt, G.E., Tobe, S.S., Weaver, R.J., 1975. Relative oxygenase activities in juvenile hormone biosynthesis of corpora allata of an African locust (*Schistocerca gregaria*) and American cockroach (*Periplaneta americana*). Experientia 31, 120–122.
Rachinsky, A., 1996. Brain and suboesophageal ganglion extracts affect juvenile hormone biosynthesis in honey bee larvae (*Apis mellifera carnica*). Zoology 99, 277–284.
Rachinsky, A., Feldlaufer, M.F., 2000. Responsiveness of honey bee (*Apis mellifera* L.) corpora allata to allatoregulatory peptides from four insect species. Journal of Insect Physiology 46, 41–46.
Rachinsky, A., Hartfelder, K., 1990. Corpora allata activity, a prime regulating element for caste-specific juvenile hormone titre in honey bee larvae (*Apis mellifera carnica*). Journal of Insect Physiology 36, 189–194.
Rachinsky, A., Hartfelder, K., 1991. Differential production of juvenile hormone and its desoxy precursor by corpora allata of honeybees during a critical period of caste development. Naturwissenschaften 78, 270–272.
Rachinsky, A., Hartfelder, K., 1998. In vitro biosynthesis of juvenile hormone in larval honey bees: comparison of six media. In Vitro Cellular and Developmental Biology — Animal 34, 646–648.
Rachinsky, A., Strambi, C., Strambi, A., Hartfelder, K., 1990. Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. General and Comparative Endocrinology 79, 31–38.
Rembold, H., 1987. Caste specific modulation of juvenile hormone titers in *Apis mellifera*. Insect Biochemistry 17, 1003–1006.
Stay, B., Tobe, S.S., Bendena, W.G., 1994. Allatostatins: identification, primary structures, functions and distribution. Advances in Insect Physiology 25, 267–337.
Stay, B., Woodhead, A.P., 1993. Neuropeptide regulators of insect corpora allata. American Zoologist 33, 357–364.
Tobe, S.S., Pratt, G.E., 1974. The influence of substrate concentrations on the rate of insect juvenile hormone biosynthesis by corpora allata of the desert locust in vitro. Biochemical Journal 144, 107–113.
Tobe, S.S., Stay, B., 1985. Structure and regulation of the corpus allatum. Advances in Insect Physiology 18, 305–432.
Trautmann, K.H., Masner, P., Schuler, A., Suchy, M., Wipf, K.-H., 1974. Evidence of the juvenile hormone methyl(2E, 6E)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (JH 3) in insects of four orders. Zeitschrift für Naturforschung C29, 757–759.
Unni, B.G., Bhaskaran, G., Dahm, K.H., Hayes, T.K., 1991. Stimulation of juvenile hormone biosynthesis by analogues of a *Manduca sexta* allatotropin: in vitro studies. Archives of Insect Biochemistry and Physiology 17, 129–142.
Unnithan, G.C., Andersen, J.F., Hisano, T., Kuwano, E., Feyereisen, R., 1995. Inhibition of juvenile hormone biosynthesis and methyl farnesoate epoxidase activity by 1,5-disubstituted imidazoles in the cockroach, *Diploptera punctata*. Pesticide Science 43, 13–19.
Unnithan, G.C., Feyereisen, R., 1995. Experimental acquisition and loss of allatostatin sensitivity by corpora allata of *Diploptera punctata*. Journal of Insect Physiology 41, 975–980.
Wang, Z., Ding, Q., Yagi, K.J., Tobe, S.S., 1994. Terminal stages in juvenile hormone biosynthesis in corpora allata of *Diploptera punctata*: developmental changes in enzyme activity and regulation by allatostatins. Journal of Insect Physiology 40, 217–223.
Weiss, K., 1978. Zur Mechanik der Kastenentstehung bei der Honigbiene (*Apis mellifica* L.). Apidologie 9, 223–258.
Wennauer, R., Hoffmann, K.H., 1988. Studies on the de novo synthesis of juvenile hormone III and methyl farnesoate by isolated corpora allata of adult female *Gryllus bimaculatus*. Insect Biochemistry 18, 867–872.
Wirtz, P., 1973. Differentiation in the honeybee larva: a histological, electron-microscopical and physiological study of caste induction in *Apis mellifera mellifera* L. Meded. Landbouwhogeschool Wageningen 73, 5.
Zdarek, J., Haragsim, O., 1974. Action of juvenoids on metamorphosis of the honey-bee, *Apis mellifera*. Journal of Insect Physiology 20, 209–221.